Hepatitis E Virus Infections in Free-Ranging and Captive Cetaceans, Spain, 2011–2022

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Epidemiologic surveillance of hepatitis E virus in over 300 free-ranging and captive cetaceans in waters off Spain revealed extensive exposure to this pathogen. We suggest the persistent and widespread presence of hepatitis E in the marine environment off the coast of Spain may be driven by terrestrial sources of contamination.

Paslahepevirus balayani (previously known as hepatitis E virus [HEV]; family *Hepeviridae*) is the leading cause of acute viral hepatitis in humans (*1*,*2*). Although 8 different genotypes of HEV have been identified, HEV-3 is the genotype with the broadest geographic distribution, including Europe, where the number of hepatitis E cases has sharply increased in the past decade (*3*). The main reservoirs of this genotype are suids, but a wide range of other land mammals has been shown to be susceptible to this emerging genotype (*2*). Although echinoderms and several bivalve shellfish species from coastal waters have tested positive for HEV, the susceptibility of other marine animals, such as cetaceans, to HEV has been unknown, as has their possible role in the epidemiology of this family of viruses (*4*). We conducted a large-scale study to determine the seroprevalence and prevalence of HEV in cetacean populations, both free-ranging and captive, in Spain, and to assess the dynamics of seropositivity in marine animals sampled longitudinally during the study period.

The Study

We collected blood and liver samples from 304 cetaceans belonging to 13 different species in Spain during 2011–2022 (Table 1; Figure 1). We based our study on 240 free-ranging animals found stranded on the Atlantic and Mediterranean coasts of Spain and 64 cetaceans kept in captivity at 6 aquatic parks (deemed A–F) in Spain. We performed longitudinal surveillance on 30 of the 64 animals kept in aquatic parks during the study period.

We assessed the presence of HEV antibodies in serum or plasma using a commercial multispecies ELISA (MP Biomedicals, https://www.mpbio.com) and, whenever possible, further investigated seropositivity by Western blot analysis (Appendix, https://wwwnc.cdc.gov/EID/article/28/12/22-1188-App1.pdf). We determined the presence of HEV RNA by using 2 broad-spectrum reverse transcription PCR (RT-PCR) assays in parallel (Appendix) (*5*,*6*). We analyzed

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Table 1. Distribution of hepatitis E virus seroprevalence in free-ranging and captive cetacean populations in Spain and results of bivariate analysis*

Variable	Categories	Free-ranging		Captive	
		No. positive/no. analyzed (% positive)†	p value	No. positive/no. analyzed (% positive)†	p value
Species‡	Atlantic spotted dolphin (*Stenella frontalis*)	1/1 (100.0)	0.191	NA	0.323
	Beluga (*Delphinapterus leucas*)	NA		0/2 (0.0)	
	Bottlenose dolphin (*Tursiops truncatus*)	0/2 (0.0)		21/55 (38.2)	
	Common dolphin (*Delphinus delphis*)	1/2 (50.0)		NA	
	Cuvier's beaked whale (*Ziphius cavirostris*)	1/1 (100.0)		NA	
	Killer whale (*Orcinus orca*)	NA		4/7 (57.1)	
	Risso's dolphin (*Grampus griseus*)	3/8 (37.5)		NA	
	Southern long-finned pilot whale (*Globicephala melas*)	0/1 (0.0)		NA	
	Striped dolphin (*Stenella coeruleoalba*)	38/57 (66.7)		NA	
Age§	Adult	33/45 (73.3)	0.006	20/47 (42.6)	0.406
	Young	11/27 (40.7)		4/12 (33.3)	
Sex	F	25/39 (64.1)	0.373	12/33 (36.4)	0.485
	M	19/33 (57.6)		12/30 (40.0)	

*Analyses by pearson's χ^2 or Fisher exact test. NA, not applicable.
†Animals with missing information excluded.
‡Samples from harbor porpoises (*Phocoena phocoena*), fin whales (*Balaenoptera physalus*), minke whales (*Balaenoptera acutorostrata*). and humpback whales (*Megaptera novaeangliae*) were also included in the study but were only tested by PCR.
§Age was classified using the mean reproductive age of each species.

associations between the presence of HEV antibodies and explanatory variables using Pearson χ^2 test or Fisher exact test and further included variables with p<0.05 in the bivariate analysis (except habitat status) in a generalized estimating equation model.

We identified 69 (50.7%, 95% CI 42.3%–59.1%) of 136 cetaceans as harboring anti-HEV antibodies (Table 1; Figures 1, 2; Appendix Table 1). We confirmed antibodies against HEV-3 in 5 of the 7 ELISA-positive animals analyzed by Western blot analysis: a free-ranging striped dolphin, a free-ranging Cuvier's beaked whale, a free-ranging Risso's dolphin, and 2 captive bottlenose dolphins. We found none (0.0%; 95% CI 0.0%–1.2%) of the 302 animals analyzed to be positive for HEV RNA (Figure 2).

We noted seroprevalence to be significantly higher in free-ranging animals (44/72; 61.1%; 95% CI 49.9%–72.4%) than in those kept in captivity (25/64; 39.1%; 95% CI 27.1%–51.0%) (relative risk = 2.5, 95% CI 1.2%–4.9%; p = 0.008). We found seropositivity in adult free-ranging cetaceans (33/45; 73.3%) to be significantly higher than that in young animals (11/27; 40.7%; odds ratio 4.0, 95% CI 1.4–11.0; p = 0.006).

Our testing revealed seropositive animals in 5 of the 6 aquatic facilities sampled; within-zoo seroprevalence ranged from 27.8% in aquatic park D to 55.6% in aquatic park E (Table 2; Figure 2). Of the 30 longitudinally sampled animals, 21 remained seronegative, and 6 animals showed seropositivity at all samplings during the study period (Appendix Table 2). Two bottlenose dolphins seroconverted, 1 in 2013 and another in 2017. Seroreversions were detected in 2 animals (1 a dolphin that had shown seroconversion); 1 incident occurred 1 year after the first positive sampling, the other 5 years.

Conclusions

Our survey reveals high exposure to HEV in free-ranging and captive populations of cetaceans in Spain. The detection of HEV antibodies in Atlantic spotted, common, Risso's, and striped dolphins, as well as in Cuvier's beaked and killer whales, demonstrates

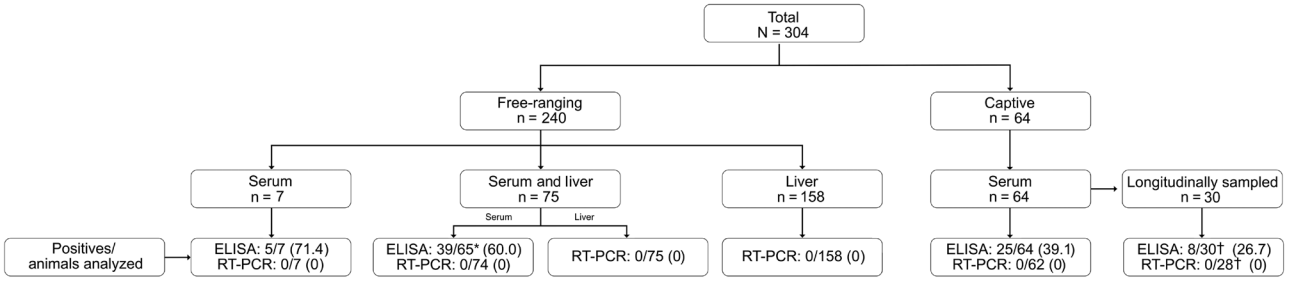

Figure 1. Flowchart of a survey of hepatitis E virus in 304 cetaceans belonging to 13 species in Spain during 2011–2022. Description of the study population, number of cetaceans, type of samples analyzed by ELISA and RT-PCR, and results obtained in each assay. *Ten of 75 serum samples were discarded for serologic analysis due to hemolysis. †Taking into account that 2–5 samples were collected per animal in longitudinally surveyed animals, 97 were analyzed by ELISA and 78 by RT-PCR. RT-PCR, reverse transcription PCR.

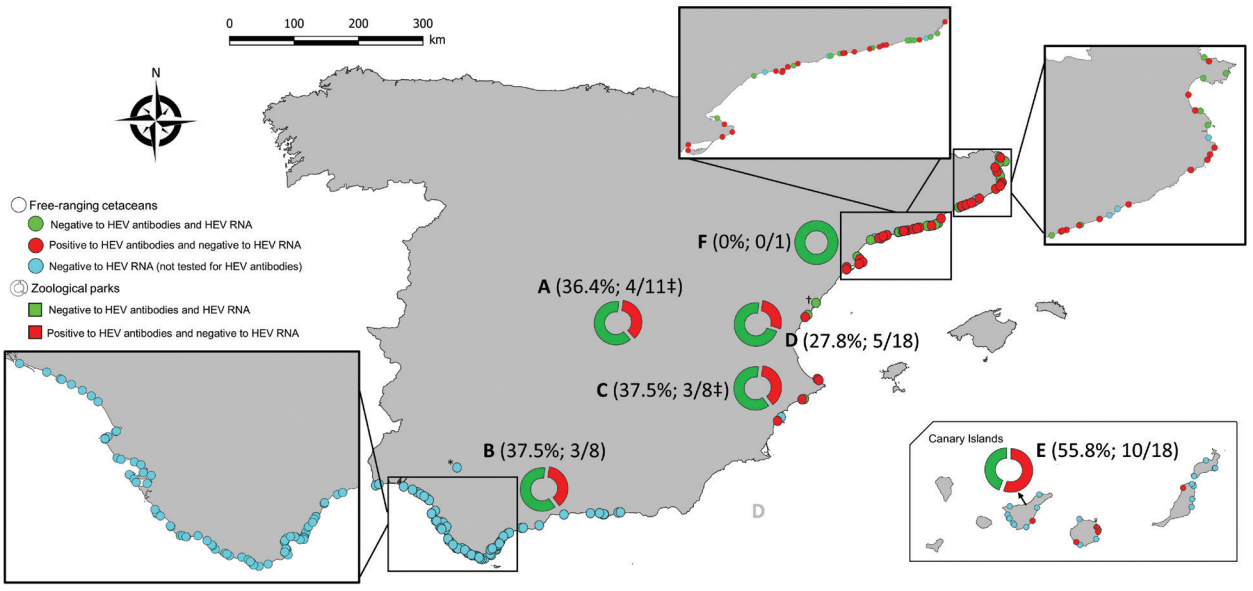

Figure 2. Spatial distribution of cetaceans sampled in a survey of HEV in 304 cetaceans belonging to 13 species in Spain during n 2011–2022. The frequency of seropositivity and number of seropositive and total animals analyzed at each zoological park (A–F) is shown in parentheses. Callouts show detail of sampling along the Atlantic and Mediterranean coastlines. *Animal sampled in the Guadalquivir River. †This animal was not analyzed by reverse transcription PCR. ‡One of the sampled animals of this zoo park was not tested by reverse transcription PCR. HEV, hepatitis E virus.

an increase in the number of cetartiodactyls susceptible to this virus (*2*).

Ingestion of contaminated food is considered to be one of the main transmission routes of HEV in humans and has also been suggested for other mammal species, including dolphins (*4*). The seropositive species detected in our study feed on a wide variety of resources, including fish and cephalopods. The presence of HEV in these food resources has not yet been assessed, but the virus has been frequently detected in such other aquatic animals as sea urchins and bivalve shellfish in different areas of Europe (*2*,*7*), which provides evidence that HEV does abide in marine ecosystems. Of note, the virus is shed primarily in the feces of infected species, which can lead to viral contamination of the environment, and HEV has been shown to be highly resistant to even high concentrations of salt (*8*). Contaminated water has been considered a potential source of zoonotic HEV (*9*), because drinking tap water or water from private wells or nearby rivers has been suggested as a risk factor for acquiring HEV infection in humans (*10*). This hypothesis is supported by a study conducted in captive cetaceans all sharing the same tanks, which revealed the detection of seropositivity and active HEV infection (*4*).

The significantly higher seroprevalence we found in adult free-ranging animals compared with young animals likely reflects the increased cumulative exposure to HEV in these species. Our additional discovery of HEV antibodies in 4 free-ranging yearlings in 2011, 2019, and 2021 could suggest endemic circulation of HEV in cetaceans living in Spanish waters during the study period. Free-ranging cetaceans had a 2.5-times higher risk of being exposed to HEV than those kept in captivity, which might be explained by differences in diet or longer exposure to environmental contamination. Human- and swine-related HEV-3 strains have been detected in sewage and slurry in Spain (*11*) and in rivers and coastal waters in Italy (*12*). The high census of some susceptible domestic and wildlife species (*13*,*14*), combined with high coastal urbanization and insufficient control of urban sewage in some regions of our study area (*15*), might be contributing factors in the higher seropositivity we noted in free-ranging cetaceans. By contrast, cetaceans in zoological parks, including those analyzed in our study, live in large

Table 2. Distribution of hepatitis E virus seroprevalence in cetaceans in Spain, by sampling location, and results of bivariate analyses

Category	No. positive/no. analyzed (% positive)	p value
Free-ranging areas		
Atlantic Ocean	6/8 (75.0)	0.327
Mediterranean Sea	38/64 (59.4)	
Aquatic parks		
A	4/11 (36.4)	0.772
B	3/8 (37.5)	
C	3/8 (37.5)	
D	5/18 (27.8)	
E	10/18 (55.6)	
F	0/1 (0.0)	

water tanks that are frequently decontaminated with ozone, ultraviolet radiation, brine, or chlorine, some of which deactivates HEV (*9*). Nonetheless, the high seroprevalence we observed in the 5 zoos with seropositive animals indicates a wide circulation of the virus in these more controlled environments.

The 2 seroconversions we noted in captive bottlenose dolphins support the hypothesis of HEV circulation in zoos during our study period. However, 4 of the longitudinally surveyed cetaceans remained seropositive at all samplings. This finding might be due to the long-lived persistence of anti-HEV antibodies in cetaceans, which is supported by the significantly higher seroprevalence we detected in older, free-ranging cetaceans. There is no known information about the long-term persistence of HEV antibodies in these species. Thus, possible loss of antibodies and reexposure in some of the persistently seropositive cetaceans during the study period cannot be ruled out, as evidenced by the seroreversions we observed in 2 bottlenose dolphins 1 and 5 years after the first seropositive sampling was detected.

In conclusion, the seropositivity noted in our study indicates widespread circulation of HEV in both free-ranging and captive cetacean populations in southwestern Europe. Additional molecular and serologic studies are warranted to determine the role of cetaceans in the epidemiology of HEV and to elucidate the sources of HEV infection, particularly in the free-ranging cetacean population.

This study did not involve the purposeful killing of animals. Samples from live cetaceans were collected from serum banks or animals in health programs or undergoing routine medical check-ups, and those from dead individuals were collected by veterinarians and animal keepers following routine procedures in compliance with Ethical Principles in Animal Research. Ethics approval by an Institutional Animal Care and Use Committee was not therefore deemed necessary.

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Appendix

Sampling

Serum or plasma samples were obtained by blood centrifugation at 400x *g* for 15 min. The median (Q1-Q3) interval between consecutive samplings of the follow-up was 35 months (range 22-118.5). Samples were stored at −20°C until laboratory analyses. Epidemiological data, including species, age, gender, habitat status (free-ranging *vs* under human care), sampling location (free-range areas [Atlantic Ocean *vs* Mediterranean Sea] and zoological institutions), sampling date and georeferenced location, were gathered from each animal. Whenever possible, both serological and molecular assays were conducted in serum samples (Table 1).

Serological analyses

The presence of anti-HEV antibodies was assessed using a commercial ELISA (HEV 4.0v; MP Diagnostics, Illkirch, France), which is based on the highly conserved and recombinant protein ET2.1 (*1*) and detects total antibodies against this virus in serum or plasma samples from a wide range of animal species. The cut-off was calculated using the formula: 0.2 + mean optical density (OD) of negative controls. In addition, sample results were expressed as an ELISA percentage (E%), calculated as follows: [E% = (sample OD)/(cut-off) × 100]. Longitudinally surveyed animals were considered seropositive if at least one serum sample tested positive by ELISA.

Whenever possible, samples from seropositive cetaceans were further investigated by western blot (WB) analysis to confirm exposure to *Paslahepevirus balayi* and/or *Rocahepevirus ratti* species, including HEV-3 and HEV-C1 genotypes, in cetaceans. In this connection,

Rocahepevirus ratti is other hepevirus whose zoonotic potential has recently been confirmed both by the detection of viral RNA in human hepatitis E cases and by experimental transmission to non-human primates (*2*,*3*). For WB analyses, carboxy-terminal segments of the capsid proteins of HEV-3 and HEV-C1, and a nucleocapsid protein derivative (amino acid residues 1-39/213-134 433) of the *Puumala orthohantavirus* strain Vranica/Hällnäs as negative control, were produced as His-tagged recombinant proteins in *Escherichia coli* and purified by nickel-chelate affinity chromatography (*4*,*5*). Seropositivity was confirmed by WB when blot bands matching either HEV-3 or HEV-C1 antigens or both were observed, but without reactivity to the negative control antigen. The presence of specific antibodies against HEV-3 or HEV-C1 was considered when samples reacted against the capsid protein derivative of only one of these genotypes, otherwise the result was considered indeterminate.

Molecular analyses

RNA was extracted from serum/plasma and liver samples using the QIAmp MinElute Virus Spin and RNeasy Mini kits (QIAGEN, Hilden, Germany), respectively. Liver RNA samples were extracted individually, whereas RNA from serum/plasma samples was obtained using pools of four samples (total volume: 400µl). A real-time RT-PCR (CFX Connect Real Time PCR System) that detects all *Paslahepevirus balayani* genotypes (HEV-1 to HEV-8) was performed using 25µl of RNA template and the QIAGEN One-Step RT-PCR kit, as previously described (*6*). The detection limit was set at 21.9 IU/mL (95% Confidence Interval (95% CI): 17.4-34.3). A nested broad-spectrum RT-PCR (Fisher Scientific Applied Biosystems SimpliAmp™) capable of detecting the four genera of hepevirus was carried out using the QIAGEN One-Step RT-PCR kit for the first round of RT-PCR, and a premixed 2X solution containing Taq DNA Polymerase, dNTPs and reaction buffer (Promega, Madison, WI, USA) for the second round (*7*). The nested RT-PCR amplicons were examined on 1.5% agarose gels stained with RedSafe™ Nucleic Acid Staining solution (iNtRON Biotechnology, Seongnam, Korea). The WHO HEV-3a reference strain (code 6329/10), supplied by the Paul-Ehrlich Institut, was included as positive control in each run of the two RT-PCR assays used.

Appendix Table 1. Anti-HEV antibody-positive samples in free-ranging cetacean populations and those under human care in Spain, 2011–2022. Samples from longitudinally surveyed animals are labeled with the same number and consecutive letters.

ID	Species	Habitat status	Sampling location	E%
1	Striped dolphin	Free-ranging	Mediterranean Sea	345,82
7	Striped dolphin	Free-ranging	Mediterranean Sea	305,18
8	Striped dolphin	Free-ranging	Mediterranean Sea	1786,45
9	Striped dolphin	Free-ranging	Mediterranean Sea	1699,60
10	Striped dolphin	Free-ranging	Mediterranean Sea	174,90
12	Striped dolphin	Free-ranging	Mediterranean Sea	1680,48
14	Risso's dolphin	Free-ranging	Mediterranean Sea	767,73
15	Striped dolphin	Free-ranging	Mediterranean Sea	958,96
16	Striped dolphin	Free-ranging	Mediterranean Sea	1821,51
18	Striped dolphin	Free-ranging	Mediterranean Sea	475,70
20	Striped dolphin	Free-ranging	Mediterranean Sea	733,86
22	Striped dolphin	Free-ranging	Mediterranean Sea	1803,19
25	Striped dolphin	Free-ranging	Mediterranean Sea	105,98
26	Striped dolphin	Free-ranging	Mediterranean Sea	1684,06
28	Striped dolphin	Free-ranging	Mediterranean Sea	381,67
29	Striped dolphin	Free-ranging	Mediterranean Sea	1647,81
30	Striped dolphin	Free-ranging	Mediterranean Sea	1882,47
33	Striped dolphin	Free-ranging	Mediterranean Sea	1715,94
35	Striped dolphin	Free-ranging	Mediterranean Sea	578,49
37	Striped dolphin	Free-ranging	Mediterranean Sea	325,50
38	Striped dolphin	Free-ranging	Mediterranean Sea	1595,62
40	Striped dolphin	Free-ranging	Mediterranean Sea	1279,68
45	Striped dolphin	Free-ranging	Mediterranean Sea	515,14
49	Striped dolphin	Free-ranging	Mediterranean Sea	288,45
50	Striped dolphin	Free-ranging	Mediterranean Sea	163,75
51	Striped dolphin	Free-ranging	Mediterranean Sea	1705,58
55	Striped dolphin	Free-ranging	Mediterranean Sea	1768,53
56	Risso's dolphin	Free-ranging	Mediterranean Sea	290,84
59	Striped dolphin	Free-ranging	Mediterranean Sea	1777,29
60	Striped dolphin	Free-ranging	Mediterranean Sea	707,57
64	Striped dolphin	Free-ranging	Mediterranean Sea	1191,63
65	Striped dolphin	Free-ranging	Mediterranean Sea	921,91
66	Striped dolphin	Free-ranging	Mediterranean Sea	1656,18
67	Striped dolphin	Free-ranging	Mediterranean Sea	778,37
69	Striped dolphin	Free-ranging	Mediterranean Sea	474,69
70	Striped dolphin	Free-ranging	Mediterranean Sea	293,06
74	Striped dolphin	Free-ranging	Mediterranean Sea	1731,43
75	Striped dolphin	Free-ranging	Mediterranean Sea	1595,92
78	Striped dolphin	Free-ranging	Atlantic Ocean	1861,84
79	Cuvier's beaked Whales	Free-ranging	Atlantic Ocean	1255,58
83	Striped dolphin	Free-ranging	Atlantic Ocean	706,07
84	Risso's dolphin	Free-ranging	Atlantic Ocean	500,59
87	Short-beaked common dolphin	Free-ranging	Atlantic Ocean	420,74
101	Atlantic spotted dolphin	Free-ranging	Atlantic Ocean	455,97
581	Bottlenose dolphin	Captivity	A	1700,60
583a	Bottlenose dolphin	Captivity	A	1136,33
583b	Bottlenose dolphin	Captivity	A	1187,35
583c	Bottlenose dolphin	Captivity	A	1102,45
587a	Bottlenose dolphin	Captivity	A	1633,06
594a	Bottlenose dolphin	Captivity	A	1598,79
594b	Bottlenose dolphin	Captivity	A	1470,20
1134	Bottlenose dolphin	Captivity	B	1006,12
1137	Bottlenose dolphin	Captivity	B	124,08
1138	Bottlenose dolphin	Captivity	B	1564,90
1187b	Bottlenose dolphin	Captivity	C	1616,33
1193a	Bottlenose dolphin	Captivity	C	220,50
1193b	Bottlenose dolphin	Captivity	C	178,72
1198	Bottlenose dolphin	Captivity	C	453,88
387a	Bottlenose dolphin	Captivity	D	117,99
392a	Bottlenose dolphin	Captivity	D	231,02
392b	Bottlenose dolphin	Captivity	D	264,49
392c	Bottlenose dolphin	Captivity	D	586,94
394a	Bottlenose dolphin	Captivity	D	225,31
394b	Bottlenose dolphin	Captivity	D	306,94
394c	Bottlenose dolphin	Captivity	D	177,14
399c	Bottlenose dolphin	Captivity	D	947,39
399d	Bottlenose dolphin	Captivity	D	389,64

ID	Species	Habitat status	Sampling location	E%
399e	Bottlenose dolphin	Captivity	D	351,84
1418b	Bottlenose dolphin	Captivity	D	233,47
1418c	Bottlenose dolphin	Captivity	D	264,60
81	Bottlenose dolphin	Captivity	E	763,64
1419	Bottlenose dolphin	Captivity	E	1144,24
1424	Bottlenose dolphin	Captivity	E	1608,48
1427	Bottlenose dolphin	Captivity	E	1397,17
1429	Bottlenose dolphin	Captivity	E	1690,91
82a	Killer whale	Captivity	E	125,64
82b	Killer whale	Captivity	E	227,47
1421	Killer whale	Captivity	E	217,37
1425	Killer whale	Captivity	E	187,47
1426	Killer whale	Captivity	E	480,00
1432	Killer whale	Captivity	E	150,30

E%, ELISA percentage; calculated as follows: [E% = (sample OD)/(cut-off) × 100]

Appendix Table 2. Antibodies against hepatitis E virus in longitudinally sampled cetaceans in Spain, 2011–2022. Dots indicate antibodies to hepatitis E virus (hollow: positive; solid: negative). When 2 samplings were carried out in the same year, abbreviated months are indicated.

ID	Species	Zoo	Interpretation	2009	2010	2013	2016	2017	2018	2019	2020	2021
383	Beluga	D	Seronegative at all samplings					●			●	
584	Bottlenose dolphin	A	Seronegative at all samplings					●		●		
586	Bottlenose dolphin	A	Seronegative at all samplings					●			●	
583	Bottlenose dolphin	A	Seropositive at all samplings					○		○	○	
594	Bottlenose dolphin	A	Seropositive at all samplings					○		○		
589	Bottlenose dolphin	A	Seronegative at all samplings				●Apr ●Jun					
595	Bottlenose dolphin	A	Seronegative at all samplings					●		●		
591	Bottlenose dolphin	A	Seronegative at all samplings					●		●		
592	Bottlenose dolphin	A/B	Seronegative at all samplings					●			●	
593	Bottlenose dolphin	A/B	Seronegative at all samplings					●			●	
1188	Bottlenose dolphin	C	Seronegative at all samplings							●	●	
1193	Bottlenose dolphin	C	Seropositive at all samplings							○	○	
1194	Bottlenose dolphin	C	Seronegative at all samplings							●		●
1199	Bottlenose dolphin	C	Seronegative at all samplings							●		●
399	Bottlenose dolphin	D	Seroconversion			●Aug ●Nov		○	○		○	
394	Bottlenose dolphin	D	Seropositive at all samplings					○	○		○	
392	Bottlenose dolphin	D	Seropositive at all samplings					○	○		○	
1418	Bottlenose dolphin	D	Seroconversion & Seroreversion	●		○		○	●		●	
387	Bottlenose dolphin	D	Seroreversion					○	●		●	
388	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
389	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
390	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
391	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	

ID	Species	Zoo	Interpretation	2009	2010	2013	2016	2017	2018	2019	2020	2021
393	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
395	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
398	Bottlenose dolphin	D	Seronegative at all samplings					●	●			
396	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
400	Bottlenose dolphin	D	Seronegative at all samplings	●		●		●	●		●	
397	Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
82	Bottlenose dolphin	E	Seropositive at all samplings									○Feb ○Jun

Apr, April; Jun, June; Aug, August; Nov, November; Feb, February

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